

**FIGURE 1**

**Minicell purification procedure without the inventive techniques**

Overnight culture of minicell producing bacterial cells



Differential centrifugation @ 2000g 20 min  
*Pellet most bacterial cells while minicells remain in supernatant*



First Cross-flow filtration through 0.45  $\mu$ m filter  
*Reduce parent bacterial cell contamination minicells are 0.4 $\mu$ m diameter & filter through but some loss of minicells*



Second Cross-flow filtration through 0.45  $\mu$ m filter  
*Further reduce parent bacterial cell contamination. Some loss of minicells.*



Cross-flow filtration through 0.2  $\mu$ m filter  
*Retain minicells but eliminate all contaminants smaller than 0.2 $\mu$ m in size*



Cross-flow filtration through 0.1  $\mu$ m filter  
*Concentrate minicells*



Dead-end filtration through 0.45  $\mu$ m filter  
*Eliminate most of the residual parent bacterial cells*



Concentrate minicells through 100kDa filter

**Inventive steps described in this patent application**

2x Density gradient centrifugation using isotonic and non-toxic density gradient media e.g. OptiPrep.  
*Removes most parent bacterial cells with minimal loss of minicells*



Cross-flow filtration through 0.45  $\mu$ m filter  
*Reduces parent bacterial cell contamination*



Stress-induced filamentation of residual parent bacterial cells e.g. incubate minicell suspension with growth media and 5% NaCl (stress inducer) for 4 hrs.  
*Residual parent bacteria turn into filaments and are blocked in subsequent filtration process*



Treat with antibiotic that parent bacteria are sensitive to.  
*Kills all live bacteria present in the preparation*



Cross-flow filtration through 0.2  $\mu$ m filter  
*Eliminates all contaminants smaller than 0.2  $\mu$ m. Dead bacterial cells, filaments and minicells are retained.*

Replace with



*Dead-end filtration also eliminates filamentous dead parent bacteria*

Incubate minicells with endotoxin removal system e.g. Anti-Lipid A antibody conjugated to magnetic beads. The free lipopolysaccharide / Anti-Lipid A conjugated magnetic beads are removed from the suspension by placing the tube in a magnetic stand to immobilise the complex and minicells can be collected.  
*This step ensures that sufficient free endotoxin is removed from the minicell suspension to enable in-vivo use of minicells in humans or animals.*

Figure 2

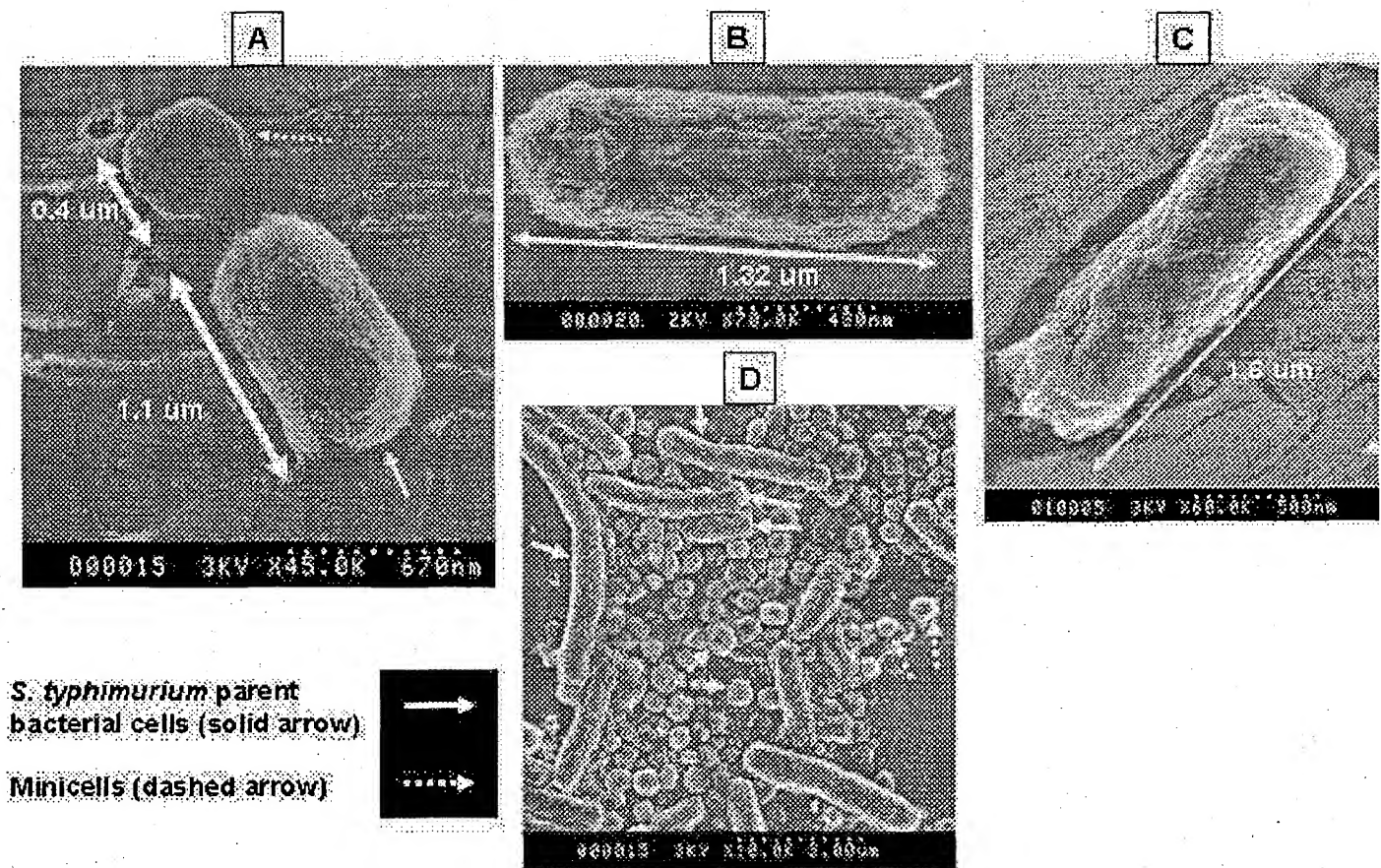
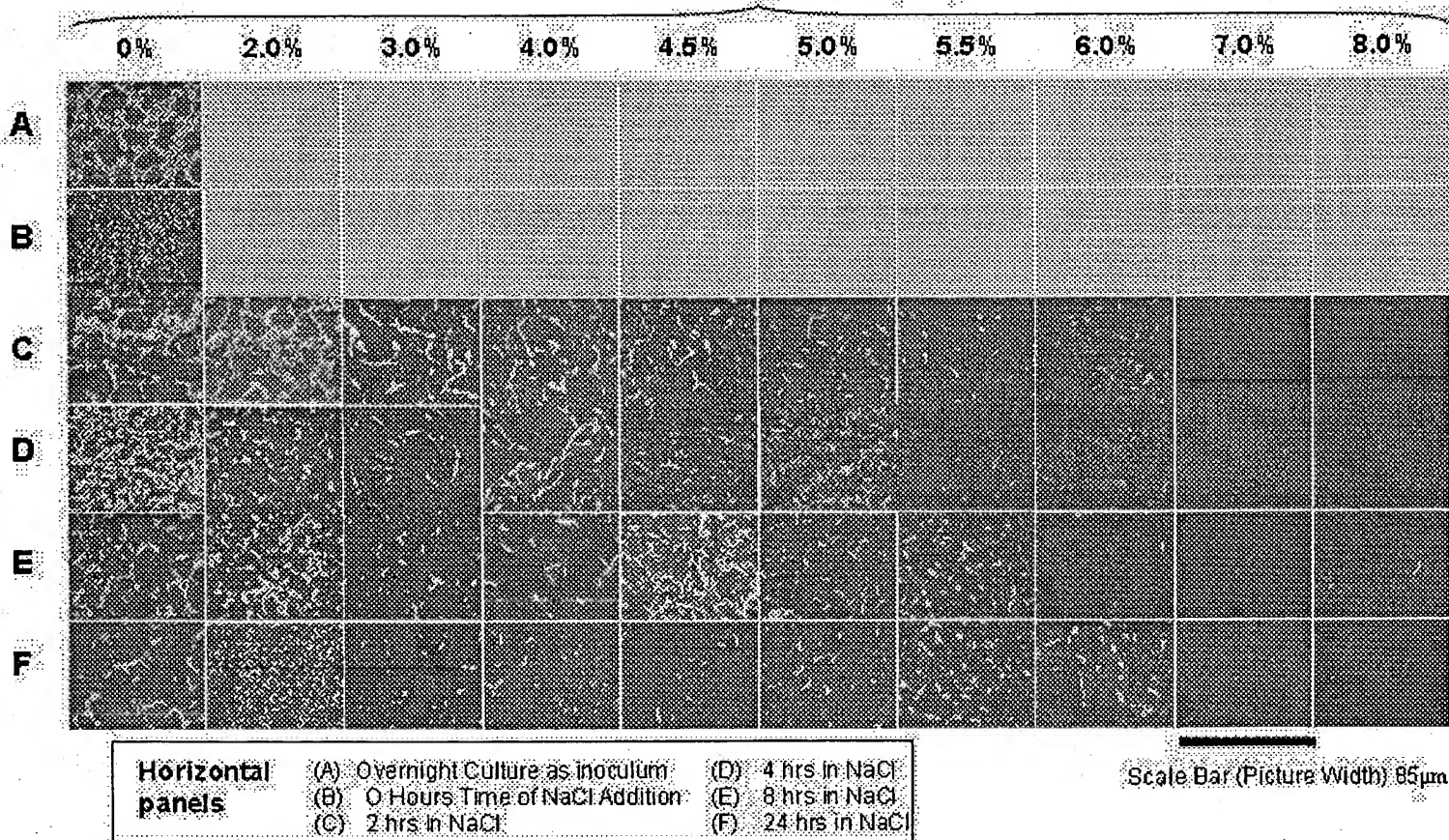


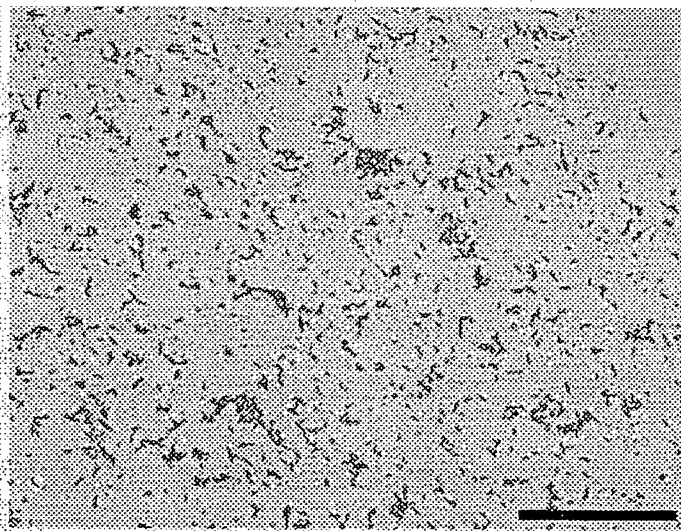
Figure 3 A

Filamentation of *S. typhimurium* [ENSm001] after addition of NaCl (1250x Magnification)  
NaCl concentration (w/v)

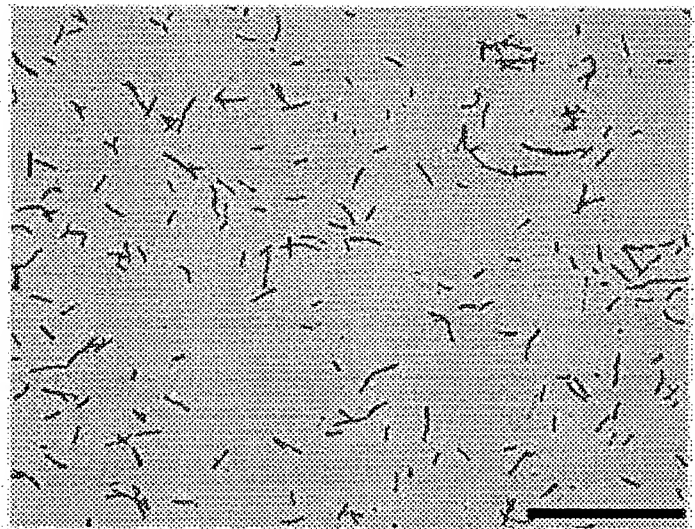


**Figure 3 B**

**Salt Induced Filamentation in *Salmonella typhimurium* ENSm026**



**4 hours after Log Culture Inoculum (0% NaCl)**

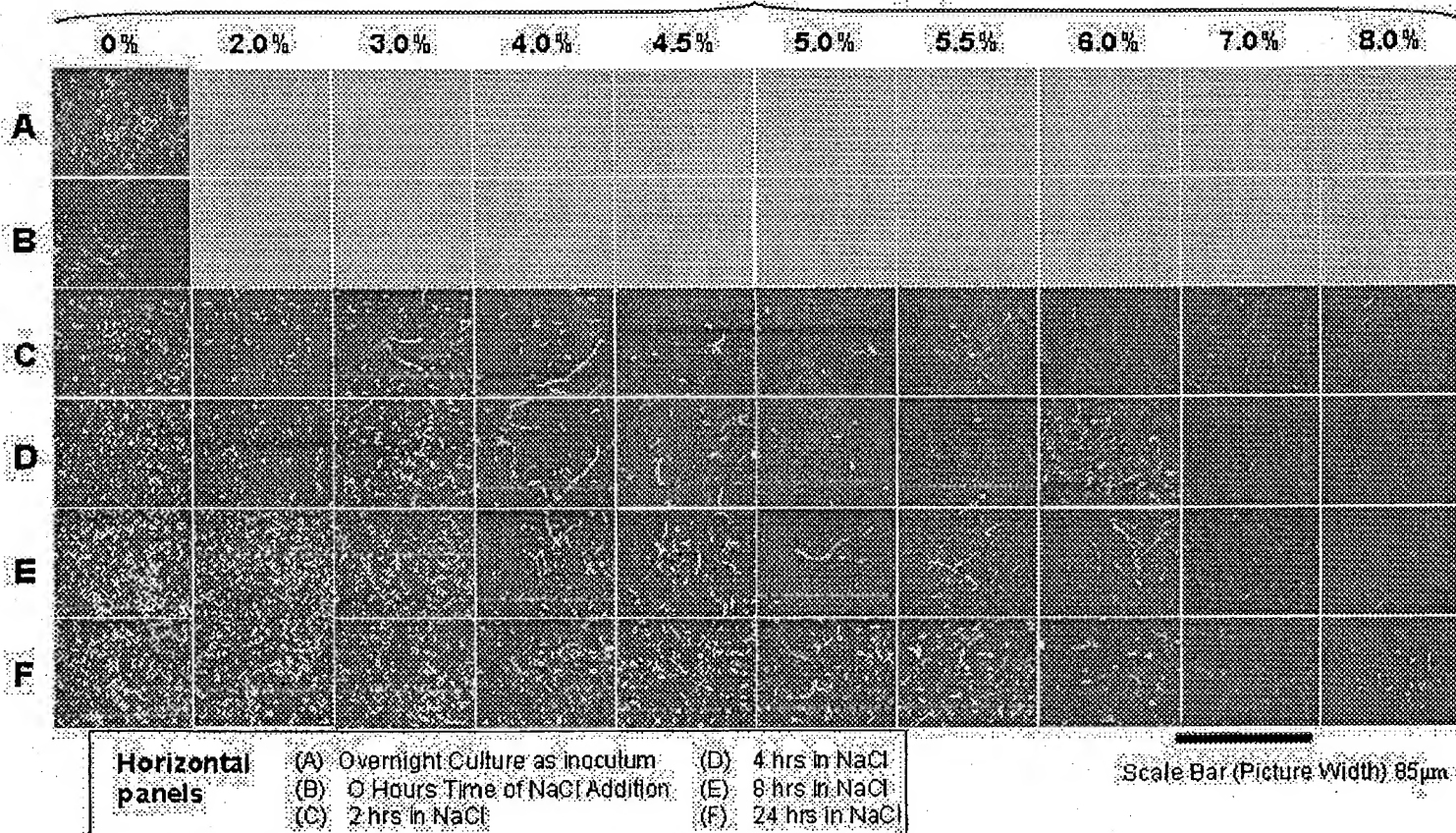


**4 hours after Log Culture Inoculum (5.0% NaCl)**

**Scale Bar 50µm**

**Figure 4 A**

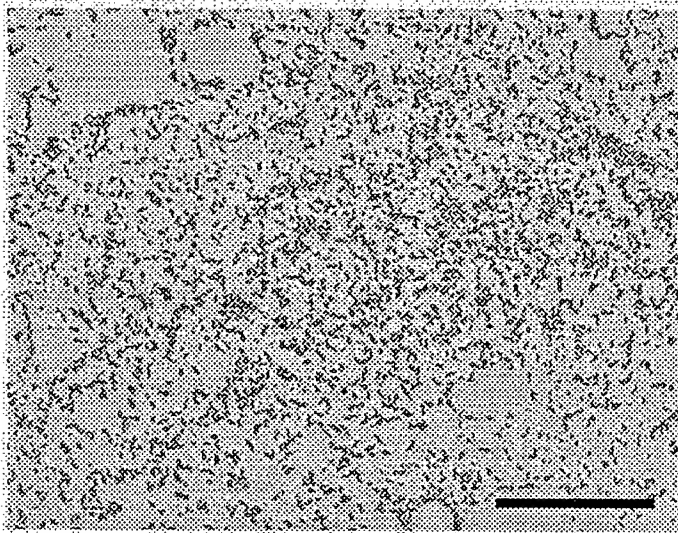
**Filamentation of *E. coli* after addition of NaCl (1250x Magnification)**  
**NaCl concentration (w/v)**



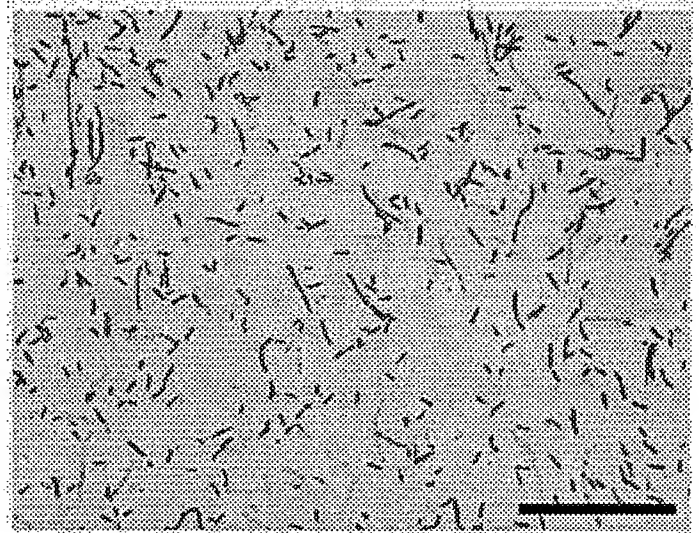


**Figure 4 B**

**Salt Induced filamentation in *E. coli***



**4 hours after Log Culture Inoculum (0% NaCl)**



**4 hours after Log Culture Inoculum (5.0% NaCl)**

**Scale Bar 50µm**